Ammine/Amine Platinum(IV) Dicarboxylates: A Novel Class of Platinum Complex Exhibiting Selective Cytotoxicity to Intrinsically Cisplatin-resistant Human Ovarian Carcinoma Cell Lines

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ABSTRACT

Using a panel of six human ovarian carcinoma cell lines varying by two orders of magnitude in terms of cisplatin cytotoxicity, we have investigated the in vitro antitumor activity of a series of novel alkyamine ammine dicarboxylato dichloroplatinum(IV) complexes of the general formula c, t, c-[PtCl2(OCOR)2]NH2(diNH2)]. A clear relationship existed between increasing the number of carbons in the R1 substituent and increasing cytotoxicity up to R1 = C6H13. In terms of changing the R2 group, maximum cytotoxic effects were conferred by alicyclic substituents. Furthermore, increasing the allicyclic ring size from cyclobutane through to cycloheptane resulted in increasing cytotoxicity. The agents with longer axial chains [e.g., JM300, R = cyclohexyl, R1 = C6H13] were significantly more cytotoxic than cisplatin and, moreover, exhibited a selective cytotoxic effect against the most intrinsically cisplatin-resistant cell lines [e.g., for HX/62, cisplatin 50% inhibitory concentration, 12.6 μM; SKOV-3, cisplatin 50% inhibitory concentration, 4.4 μM and 41 μM; cisplatin 50% inhibitory concentration, 0.23 μM; JM300 was 840-, 440- and only 34-fold more active, respectively]. The dicarboxylates JM221 (R = cyclohexyl, R1 = C6H13) and JM244 (R = n-propyl, R1 = C6H13) also retained activity against a 4-fold cisplatin-acquired resistant variant of the 41M cell line. At least part of the increased cytotoxicity of the dicarboxylate, JM221, over cisplatin appeared to be attributable to an increased intracellular accumulation. This novel class of platinum compound represents a valuable lead in the development of a “third-generation” agent capable of exhibiting activity against clinical disease currently resistant to cisplatin.

INTRODUCTION

The introduction of the square-planar complex cisplatin [cis-diaminedichloroplatinum(II)] into the clinical treatment of cancer has resulted in dramatic improvements in the response rate for some tumor types, notably testicular teratoma and ovarian carcinoma (1, 2). While the unfavorable toxicity profile of cisplatin (nephrotoxicity) has been overcome by the development of the second-generation agent, carboplatin (3–5), there remains an unquestionable need for further platinum drugs which circumvent resistance. As with other cancer chemotherapy agents, cellular resistance to the clinically used platinum agents, cisplatin and carboplatin, represents a major clinical limitation to their efficacy (6–8). Indeed, a recent United States survey has revealed that, of approximately 18,000 new ovarian cancer cases diagnosed per annum, two thirds will ultimately die of their disease (9).

Recent clinical data have shown a low (around 6%) statistically nonsignificant incidence of non-cross-resistance between cisplatin and carboplatin, suggesting that these two agents are effective against essentially the same population of tumors (10, 11). Moreover, a similarly low level of non-cross-resistance among cisplatin, carboplatin, and irinotecan has been reported (12, 13).

Tumor cell resistance to cisplatin/carboplatin may either be present at the onset of treatment (intrinsic) or be acquired during subsequent treatments. Laboratory-based biochemical studies, using a variety of murine and human tumor models, point to a multifocal basis for resistance to platinum drugs involving one or more of transport, intracellular detoxification, chromatin binding, and DNA repair mechanisms (14, 15). Furthermore, it is largely unclear whether intrinsic resistance and acquired resistance are due to similar or dissimilar mechanisms.

To date, there has been a notable paucity of novel platinum chemistry addressing the issue of resistance. Perhaps the only prominent contribution thus far, in over 20 yr of study, has been the discovery of the 1,2-diaminocyclohexane/heptane carrier ligand (16). These DACH3 complexes retain activity in cisplatin-acquired resistant murine leukemias, both in vitro (17, 18) and in vivo (19). However, using tetraplatin [(trans-d,l)-1,2-diaminocyclohexane tetrachloroplatinum(IV)], previous studies in this Department have shown no activity against another cisplatin-acquired resistant murine model the ADJ/PC6 plasmacytoma (20).

Moreover, across 16 human ovarian carcinoma xenografted tumors, only two were sensitive to tetraptalin, these being the same tumors which were particularly sensitive to cisplatin and carboplatin (21). At present the clinical relevance of the DACH carrier ligand in platinum complexes is largely untested. Some DACH complexes [e.g., 1,2-diaminocyclohexane(4-carboxyphthalato)platinum(II), JM82] and the closely related TN0-6 [1,1-diaminomethylcyclohexane sulfatoplatinum(II)] have entered Phase I/II clinical trials but have subsequently been dropped, primarily due to unacceptable toxicity (22–25).

Currently, two more water-soluble DACH complexes, oxalatoplatinum(1,2-diaminocyclohexane oxalatoplatinum(II)) (26) and tetraptalin (27) are undergoing early clinical evaluation.

Our platinum-based drug discovery program, is aimed at developing drugs capable of broadening the clinical spectrum of activity of cisplatin/carboplatin through circumvention of cisplatin resistance. To assist in these objectives we have established human ovarian tumor models [both in vitro (28) and in vivo (21)] containing examples of both intrinsic and acquired resistance to cisplatin. In the present study, a subpanel of the in vitro cell lines has been used to evaluate the cytotoxic properties of a novel class of platinum(IV) complex, namely, ammine/amine dicarboxylates.
MATERIALS AND METHODS

Cell Lines

Six "parent" human ovarian carcinoma cell lines have been used. SKOV-3 (29) and OVCAR-3 (30) were obtained from the American Type Culture Collection. Establishment details and biological properties of the other four cell lines (HX/62, PXN/94, CH1, and 41M) have been described previously (28). In addition, acquired resistance to cisplatin has been developed in the 41M cell line by continuously exposing cells to increasing concentrations of drug (up to a maximum of 1 µM) over a 15-mo period. A cell line, 41M cisR, was approximately 6-fold resistant to cisplatin in IC50 terms when compared with the parent line.

All seven cell lines grew as monolayers in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (Imperial Laboratories, Andover, United Kingdom), 50 µg/ml of gentamicin, 2.5 µg/ml of amphotericin B, 2 mM L-glutamine, 10 µg/ml of insulin, and 0.5 µg/ml of hydrocortisone in 10% CO2/90% air. Cells were free from Mycoplasma contamination throughout the course of the study.

Platinum Agents

Platinum-containing agents were all synthesized by and obtained from the Johnson Matthey Technology Centre (Reading, Berkshire, United Kingdom) and the Johnson Matthey Biomedical Research Centre (West Chester, PA). Details of the synthesis of these agents have been described recently (31). The generalized structure of the platinum(IV) dicarboxylates and the structures of other platinum agents used in this study are shown in Fig. 1.

Assessment of Cytotoxicity

Platinum agents were dissolved immediately before use in 0.9% saline (for cisplatin, JM118, and JM149) or water (for carboplatin) or absolute ethanol [for the platinum(IV) dicarboxylates]. Where ethanol was used, the final concentration of solvent in the growth medium was 0.5%; this concentration had no growth-inhibitory effect and, moreover, the cytotoxicity of cisplatin was unaltered. Three independent methods of assessing cytotoxicity were adopted. In all cases, cell lines were used within a defined passage range of 20; during this period no differences in population doubling time or morphological appearance were apparent for any of the lines.

Clonogenic Assay. The colony-forming assay was performed as described previously (32). Briefly, single cells, harvested using a 5 min treatment with 0.25% trypsin and 0.02% EDTA, were seeded into triplicate 25 cm2 tissue culture flasks, each containing 4.5 ml of growth medium. After overnight attachment at 37°C, cells were exposed to varying concentrations of platinum agent (added in 0.5 ml as a 10× concentrate). After 10 to 12 days, the flasks were washed with PBS and stained for 10 min using 0.5% methylene blue in 50% methanol/water, and colonies containing greater than 50 cells were counted by eye. Under these conditions the SKOV-3 cell line typically gave colony-forming efficiencies in the region of 30%. Accumulation studies were performed with cisplatin [cis-diammine-dichloroplatinum(II)] and JM221 [(ammine dibutyratodichloro(cyclohexylamine)platinum(IV)] using the SKOV-3 cell line. Agents were added for 2 h at various concentrations to approximately 3 × 103 cells growing exponentially. Immediately after exposure, cell monolayers were washed 3 times with ice-cold PBS, scraped, and harvested in 0.5 ml of PBS. Samples, held on ice, were then sonicated (Soniprep 150; Fisons, Loughborough, United Kingdom). Total intracellular platinum content was then determined by flameless atomic absorption spectroscopy (Perkin Elmer Models 1100B and HGA700). Protein content was analyzed according to the method of Lowry et al. (34). Cellular platinum levels were expressed as nmol of platinum/mg of protein.

RESULTS

Cytotoxicity determinations for cisplatin, carboplatin, JM118, JM149, and three ammine/amine platinum(IV) dicarboxylates (JM221, R = cyclohexyl, R1 = n-C6H13; JM274, R = cyclohexyl, R1 = n-C6H13; JM244, R = n-propyl, R1 = C6H13) against a panel of six human ovarian carcinoma cell lines are shown, using the sulforhodamine B assay (Fig. 2). It is apparent that, for all six lines, the three ammine/amine platinum(IV)
dicarboxylates confer substantially greater cytotoxicity than the other compounds, including cisplatin.

Structure-Activity Relationships. We have investigated the effect on cytotoxicity of substitutions of both the R (amine) and R₁ (axial) ligands using the sulforhodamine B assay. Table 1 shows the effect of various amine ligand substitutions for a series of complexes where the axial ligand is acetato (R₁ = CH₃). Examples of straight- and branched-chain aliphatic, alicyclic, and aromatic groups are included. The table identifies the following features: (a) the parent diammine (JM211) was significantly less cytotoxic than the mixed amine complexes where R = aliphatic or alicyclic; (b) for the mixed amine complexes, cytotoxicity increased from aromatic (e.g., JM281 and JM285), to straight chain aliphatic (JM189), to branched chain aliphatic, through to alicyclic groups conferring greatest cytotoxicity; (c) for the alicyclic mixed amines where R varied from cyclobutyl (JM236) to cycloheptyl (JM269), cytotoxicity generally increased with each stepwise increase in alicyclic ring size.

Table 2 shows a similar series of complexes where R₁ = n-
C₂H₇; i.e., the axial ligand is butyrate. On comparison with the corresponding R substitution, for the acetato complexes shown in Table 1, it is clear that the butyrates confer greater cytotoxicity (e.g., where R = cyclohexyl, JM221, the dibutyrate, was an average of 14-fold more cytotoxic across the six cell lines than was JM216, the corresponding diacetate). Otherwise, the structure-activity relationships observed with the acetato complexes (Table 1) are retained for the butyrates. Again, complexes possessing an alicyclic ring (particularly cyclohexyl and cycloheptyl) confer the greatest cytotoxicity.

The observation from Tables 1 and 2 that, for identical amine ligands, the butyrate was more cytotoxic than the acetate has been extended in Fig. 3. The figure shows the relationship between cytotoxicity (continuous exposure, SRB assay; mean IC₅₀ calculated for the six ovarian carcinoma cell lines) and the total number of carbons present in both of the axial chains of a series of ammine/amine platinum(IV) dicarboxylates where R = alicyclic; e.g., C₃H₇, C₄H₈, C₅H₁₀, and C₆Hn. Points, mean; bars, SD.

Also included are data for JM118 and JM149. A feature observed with both assays is the selective cytotoxicity of the dicarboxylates (JM221, JM274, JM300, and JM244) to the most intrinsically cisplatin-resistant cell lines, HX/62 and SKOV-3. This is particularly apparent on comparison of enhancement factors for HX/62 and SKOV-3 with 41M. For example, for results obtained by the sulforhodamine assay and JM300 (R = C₃H₁₁, R₁ = C₆H₁₃), enhancement factors of 840 and 440 were obtained for the HX/62 and SKOV-3 lines, respectively, whereas for 41M, the value was only 34. The selective cytotoxic effects, although still substantial, were not as pronounced with the second, relatively sensitive line, CH1, as with 41M.

Cytotoxicity of JM221 as Assessed by Colony-forming Assay. The in vitro potency of the platinum(IV) ammine/amine dicarboxylates observed with the short-term (4-day) [³H]thymidine and SRB assays has been confirmed using a colony forming end point. As neither the [³H]thymidine incorporation assay (which measures ongoing DNA synthesis) nor the SRB assay (which measures cellular basic amino acid content) at 4 days post-agent exposure distinguishes between viable and doomed cells, it was important to ensure that the effects observed with the platinum(IV) dicarboxylates were also apparent using a full 12-day clonogenic cell survival end point. Fig. 5 shows full survival curves for the SKOV-3 line for continuous exposure to cisplatin and JM221. As for the other assays, the dicarboxylate was evidently more cytotoxic; the IC₅₀ values were 1.2 µM for cisplatin and 0.02 µM for JM221 (enhancement factor of 60).

Activity of Platinum(IV) Ammine/Amine Dicarboxylates against a Cisplatin-acquired Resistant Cell Line. Fig. 6 shows resistance factors (IC₅₀ acquired resistant line/IC₅₀ parent line) for 41M cell lines. 41McisR was approximately 4-fold resistant to cisplatin and showed cross-resistance (2.8-fold) to carboplatin. The two dicarboxylates, however, (JM221 and JM244) both circumvented cisplatin acquired resistance in this model producing resistance factors lower than 1 (0.61 and 0.56, respectively).

Intracellular Accumulation of JM221 versus Cisplatin. As a preliminary insight into the possible mechanistic reasons for
The observed enhanced cytotoxicity of the longer axial chain platinum(IV) ammine/amine dicarboxylates, we have studied intracellular accumulation of JM221 versus cisplatin using the relatively cisplatin-resistant SKOV-3 cell line. Fig. 7 shows that, after a 2-h exposure to agent, at equimolar concentrations, a substantially greater level of platinum is present in cells after JM221 compared with cisplatin. At concentrations from 5 to 25 μM, there was a 50- to 100-fold increase in the levels after JM221. Cellular platinum accumulation was linear in the SKOV-3 cells up to 25 μM JM221 (r = 0.998) and up to 90 μM cisplatin (r = 0.992) exposure. At equitoxic doses of 10 μM JM221 and 40 μM cisplatin (2-h IC₅₀ values, SRB assay, were 9.7 ± 3.1 μM and 39 ± 9.7 μM, respectively), cellular platinum levels were approximately 10-fold greater after exposure to JM221 than cisplatin. Even after the highest dose used of 100 μM, however, cells remained intact throughout the exposure period as assessed by trypan blue exclusion.

**DISCUSSION**

A novel class of platinum(IV) complex, the ammine/amine platinum(IV) dicarboxylates, has been evaluated for antitumor activity against a panel of human ovarian carcinoma cell lines varying by two orders of magnitude in their sensitivity to cisplatin. Some of the dicarboxylates, notably those containing a total of at least 8 carbons in their axial ligands, exhibited particularly dramatic in vitro cytotoxicity properties. (a) In terms of potency, these agents were at least 100 times more cytotoxic than cisplatin. This has been confirmed by three independent cytotoxicity assays. (b) They appeared to exert some selectivity in their antitumor effects against the most intrinsically cisplatin-resistant cell lines (HX/62 and SKOV-3). Effectively, the longer axial chain dicarboxylates enhanced the sensitivities of the highly cisplatin-refractory HX/62 and SKOV-3 cell lines to levels above those of cisplatin in the highly sensitive 41M and CH1 lines. As platinum analogues to date have been hard pressed to match the cytotoxicity of cisplatin itself, let alone exceed it, these complexes are of exceptional interest. Moreover, the dicarboxylates were able to circumvent acquired resistance to cisplatin in the 41M cell line.

A further feature of members of the ammine/amine platinum(IV) dicarboxylate class of complex is that they may be chemically modified at a number of sites. For example, substitutions in the amine, trans-axial ligands, and leaving groups are possible. The structure-activity relationships investigated in this study have shown that the dramatic cytotoxicity effects are mediated largely through the lipophilicity of the axial ligands. However, cytotoxicity may also be mediated, but to a lesser degree, through alterations in the amine ligand. In particular, alicyclic, rather than aliphatic or aromatic, substitutions produced the greatest cytotoxic effects. Furthermore, within the alicyclic series, cyclobutane, -pentane, -hexane, and -heptane, cytotoxicity increased with each step up in ring size. To date, the effect of varying the dichloro leaving groups has not been investigated.

In order to assess fully the potential of this class of agent as a broader spectrum platinum drug, it is desirable to elucidate the mechanistic basis for the observed potency and selectivity effects. It is widely accepted that cisplatin exerts its cytotoxicity through binding to DNA (35). However, as the ammine/amine dicarboxylates are platinum(IV) complexes, previous studies with other platinum(IV) complexes would suggest that they are essentially inert to ligand substitution. For example, studies with cis-dichloro-trans-dihydroxy-bis-isopropylamine platinum(IV) (iproplatin) have identified the divalent cis-dichloro-
In summary, the potency and selectivity toward intrinsically cisplatin-resistant cell lines exhibited by this novel class of platinum complex suggest that it provides a valuable lead in the search for new broad-spectrum platinum drugs. Further studies are warranted to determine their activity in vivo in the cisplatin resistance setting and to elucidate whether the selectivity effects are mediated via tumor-dependent metabolism. As the basic structure of this novel class of platinum(IV) complex is readily amenable to chemical modification, it might be possible to further refine the enhanced selectivity effects and delineate tumor cell cytotoxicity from host toxicity.

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